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The role of the PCM in reducing oxidative stress induced by radical initiated photoencapsulation of chondrocytes in poly(ethylene glycol) hydrogels

N. Farnsworth, C. Bensard, S.J. Bryant*

Department of Chemical and Biological Engineering, University of Colorado Boulder, Boulder, CO 80309, USA

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SUMMARY

Objective: The objectives for this study were to determine whether radical initiated photopolymerizations typically employed for cell encapsulations lead to oxidative stress incurred by chondrocytes and whether the development of a pericellular matrix (PCM) decreases this oxidative stress and has longer-term benefits on chondrocyte function.

Methods: Freshly isolated bovine chondrocytes were encapsulated in poly(ethylene glycol) (PEG) hydrogels devoid of a PCM or with a PCM, confirmed by immunocytochemistry (IC), and cultured for up to 2 weeks. Reactive oxygen species (ROS) production and damage to cell membrane by lipid peroxidation were accomplished using carboxy-2,7-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) and by malondialdehyde (MDA) content, respectively. Gene expression and proteoglycan synthesis were analyzed using reverse transcription (RT)-quantitative PCR (qPCR) and $^{35}SO_4$ incorporation, respectively.

Results: The photopolymerization reaction, which alone generates radicals and extracellular ROS, led to oxidative stress in chondrocytes evidenced by increased intracellular ROS and lipid peroxidation. The presence of a PCM decreased intracellular ROS and abrogated membrane lipid peroxidation, improved aggrecan, collagen II and collagen VI expression, and enhanced proteoglycan synthesis.

Conclusions: The development of the PCM prior to photoencapsulation in PEG hydrogels reduces oxidative stress and improves chondrocyte anabolic activity. Our data suggest this reduction occurs by decreased ROS diffusion into the cell and decreased membrane damage. Our findings suggest that minimizing oxidative stress, such as through the presence of a PCM, may have long-term beneficial effects on tissue elaboration when employing photopolymerizations to encapsulate chondrocytes for cartilage tissue engineering applications.

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Introduction

Hydrogels have been widely used as platforms for three-dimensional culture of chondrocytes because they promote a rounded cellular morphology that maintains the chondrocyte phenotype, have high water contents for facile nutrient diffusion, and encourage cartilage-like tissue deposition.[1,2] Hydrogels formed through photopolymerization are of particular interest because this process affords spatial control over the polymerization, occurs rapidly on clinically relevant timescales, and can be performed at physiological temperature with minimal heats of reaction.[2–4] Moreover, photopolymerizable hydrogels can be formed from a wide range of natural and synthetic polymer precursors[5,6] to produce gels tailored to mimic native cartilage.[7]

While photopolymerization has many desirable attributes for cell encapsulation and *in vivo* delivery of cells, reduced cell viability post-encapsulation has been reported.[8–10] Because photopolymerizations are radical initiated chain polymerizations, the presence of radicals can generate a harsh environment. During photopolymerization, photoinitiator molecules absorb photons of light energy and dissociate into radicals. Initiator radicals react with functional groups on macromolecular monomers forming macroradicals that leads to chain propagation.[5,11] Initiating radicals and macroradicals may attack cells causing direct or indirect damage and ultimately leading to cell death. In addition, initiating radicals have a high propensity to react with oxygen that is present during photoencapsulation of cells, having reaction rate constants five orders of magnitude greater for oxygen over typical monomers.[12] When radicals react with oxygen, common byproducts include peroxy radicals,[13,14] a type of reactive oxygen species (ROS) known to trigger oxidative stress in cells.[15–17] In chondrocytes, oxidative stress has been linked to cell death,[17]

* Address correspondence and reprint requests to: S.J. Bryant, Department of Chemical and Biological Engineering, University of Colorado Boulder, ECCH 111 UCB 424, Boulder, CO 80309, USA. Tel: 1-303-735-6714; Fax: 1-303-492-4341.
E-mail addresses: Stephanie.Bryant@colorado.edu, sbryant@colorado.edu (S.J. Bryant).

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inhibition of tissue synthesis, and upregulation of tissue degrading enzymes, e.g., matrix metalloproteinase-13 (MMP-13).[18,19] Therefore, photoencapsulation may also adversely affect cells through polymerization-induced ROS, leading to oxidative stress in cells and ultimately reducing tissue regeneration capabilities in photopolymerized hydrogels.

Previous studies from our group have shown that chondrocyte death resulting from photoencapsulation can, in part, be mitigated by allowing cells to re-form some of their own pericellular matrix (PCM) prior to encapsulation.[20] These findings suggest that the PCM is able to protect chondrocytes possibly by reducing damage from radicals and ROS. To better understand the photopolymerization process and the protective role of the PCM, this study aimed to address three research questions. (1) Does the photopolymerization process, under conditions employed for cell encapsulations, lead to oxidative stress incurred by chondrocytes? (2) Does the presence of a PCM alleviate oxidative stress incurred by chondrocytes during photoencapsulation? and (3) Does oxidative stress impact chondrocytes over short and longer-term cultures? Specifically, markers for oxidative stress were measured by chondrocyte-generated intracellular ROS and damage to the cell membrane. Our model was bovine articular chondrocytes encapsulated in poly(ethylene glycol) (PEG) hydrogels formed from PEG dimethacrylate (PEGDM) precursors. Longer-term, chondrocyte anabolic activity was evaluated by aggrecan, collagen II and collagen VI gene expression and proteoglycan synthesis and chondrocyte catabolic activity was assessed by MMP-13 gene expression.

Materials and methods

Chondrocyte isolation

Articular cartilage from metacarpophalangeal joints of 1–2-year-old steers (Arapahoe Foods, Lafayette, CO) was harvested several hours after slaughter under sterile conditions. Cartilage was processed in one of two ways: (1) cartilage from three different animals was harvested separately and cells from a single donor were used to give three biological replicates (referred to as single donor chondrocytes) or (2) cartilage was harvested from four to six animals in two separate isolations and for each isolation the cells were pooled to give two biological replicates (referred to as pooled donor chondrocytes). All solutions were adjusted to a physiological osmolarity of 415 ± 14 mOsm by supplementation with 3.64 g/L NaCl and 0.45 g/L KCl determined by freezing point osmometry (Precision Systems Inc, Natick, MA). Cartilage slices were washed in phosphate buffered saline (PBS) with 1% penicillin/streptomycin (PBS + P/S, Invitrogen, Carlsbad, CA), diced finely, and enzymatically digested in 0.2% collagenase type II (Worthington Biochemical, Lakewood, NJ) in Dulbecco's Minimal Essential Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) for 16 h at 37°C on a shaker. Isolated chondrocytes were washed twice in PBS + 0.02% ethylenediaminetetraacetic acid (EDTA) and resuspended in PBS + P/S. Cell viability was 90–96% determined by trypan blue exclusion.

Chondrocyte studies in suspension and encapsulated

Chondrocytes were pre-cultured under non-plated or plated conditions (Fig. 1). Non-plated condition describes cells that were used immediately after enzymatic digestion. Plated condition describes cells that after enzymatic digestion were plated in two-dimensional culture at 10 million cells/10 mL standard chondrocyte medium (defined below) per dish (100 mm diameter non-tissue culture treated) for 24 h at 37°C, enabling chondrocytes to re-form some of their native PCM. Cell viability after plating was 83–96% by trypan blue exclusion. Chondrocytes were investigated in suspension cultures (details provided below) or encapsulated in PEG hydrogels.

For cell encapsulations, PEGDM was synthesized by reacting linear PEG (3,000 MW, Fluka, Milwaukee, WI) with methacryloyl chloride (97% pure, Sigma–Aldrich) in the presence of triethylamine (Sigma–Aldrich) at a molar ratio of 1:4.4:4 at 60°C for 24 h. PEGDM was purified by filtration over alumina powder and precipitated in cold ethyl ether. ^{1}H NMR analysis confirmed 75% of end hydroxyls were methacrylated by comparing the area under

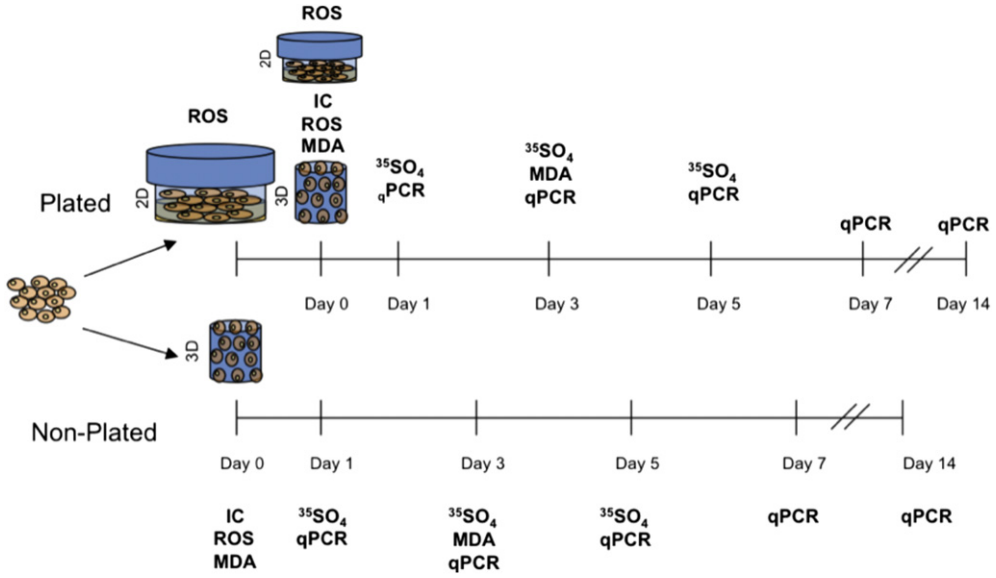

Fig. 1. The experimental design. Freshly isolated primary bovine chondrocytes were cultured under one of two conditions, referred to as non-plated or plated. Under plated conditions, chondrocytes were cultured in 2D on non-tissue culture treated dishes for 24 h and subsequently used in 2D studies to assess intracellular ROS generation or encapsulated in PEG hydrogels. Under non-plated conditions, chondrocytes were immediately used after isolation in 2D studies to assess intracellular ROS generation or encapsulated in PEG hydrogels. In 3D, cell-laden hydrogels were analyzed by IC, MDA quantification for lipid peroxidation, ^{35}Sulfate incorporation ($^{35}SO_4$) into new synthesized proteoglycans, and gene expression by qPCR at the time points indicated.

the vinyl group peaks (~5.6 and 6.1 ppm) to that of the methylene group peaks (~3.6–4 ppm). Chondrocytes (4×10^6/mL) from non-plated and plated conditions were combined with 10% w/v PEGDM and 2 mM photoinitiator (Irgacure 2959, Ciba Specialty Chemicals, Tarrytown, NY) in standard chondrocyte medium (DMEM with 4 g/L $CaCO_3$, 5.24 g/L NaCl, 0.26 g/L KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 0.1 M non-essential amino acids, 0.4 mM L-proline, 50 mg/L L-ascorbic acid, 1% P/S, 10% FBS, 0.02 mg/mL gentamicin and 0.5 μg/mL fungizone (HEPES, non-essential amino acids, gentamicin: Invitrogen, others from Sigma–Aldrich)). This solution was exposed to 365 nm light (~4 mW/cm^2) for 10 min to form stable hydrogels. Chondrocytes (4×10^6 cells/mL) from non-plated conditions were combined with 3% (w/v) agarose type IX-A (Sigma–Aldrich) at 37°C, which was previously dissolved in Earl's Balanced Salt Solution (Invitrogen) at 60°C. Gelation occurred by reducing the temperature to 4°C for 20 min. Cylindrical hydrogels (4.5 mm height, 4.5 mm diameter) were formed. Hydrogels were cultured in standard chondrocyte medium for up to 2 weeks at 37°C and 5% CO_2. Cell viability, determined by LIVE/DEAD® (Invitrogen) analysis post-encapsulation was similar between non-plated and plated conditions throughout the 2 weeks of culture (data not shown).

PCM development and visualization

PCM was assessed by immunocytochemistry (IC) for collagen II, collagen VI, and chondroitin sulfate, the main constituents of the PCM in cartilage.[21,22] Pooled donor chondrocyte-seeded PEG constructs were pre-treated with 2,080 U/mL hyaluronidase (Sigma–Aldrich) and 500 mU/mL chondroitinase-ABC (Sigma–Aldrich) in PBS with 1% bovine serum albumin (Sigma–Aldrich) for 30 min at 37°C. Constructs were treated with anti-collagen II (US Biologicals, C7510-20F, Swampscott, MA), anti-collagen VI (Abcam, ab6588, Cambridge, MA) or anti-chondroitin sulfate (Chemicon International, MK302, Temecula, CA) in DMEM + 20% FBS (1:50) for 1 h at 37°C. Constructs were treated with secondary antibodies at 1:100 (AlexaFlour546 goat anti-mouse IgG, Invitrogen) or 1:2,500 (Dylight549 goat anti-rabbit IgG, Rockland Immunochemicals, Gilbert, PA) in DMEM + 20% FBS for 1 h at 37°C. Cytosol was counterstained using 8 μM calcein AM (Invitrogen) in PBS for 30 min at 37°C. Cells and PCM were imaged using confocal laser scanning microscopy (Zeiss LSM 510, Thornwood, NY). One biological replicate was performed with two technical replicates ($n = 1$).

Quantification and visualization of ROS production using carboxy-2,7-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA)

The cell-permeant dye, carboxy-H_2DFFDA (Invitrogen) was used to quantify and visualize radicals and ROS extracellularly and intracellularly.[23,24] Once the diacetate is hydrolyzed, carboxy-H_2DFF can react with ROS (e.g., hydroxyl radicals, peroxyl radicals, superoxide anions, and hydrogen peroxide) to produce a stable fluorescent product.[23,24] In the absence of cells, the diacetate group was hydrolyzed with 200 μM KOH for 1 h.[25] The hydrolyzed dye was added at a final concentration of 20 μM to chondrocyte medium containing 0, 1, 2, 4.5 or 11 mM photoinitiator. Wells were either left untreated or exposed to 365 nm light (~4 mW/cm^2) for 10 min and fluorescence immediately assayed on a FLUOstar Optima plate reader (BMG Labtech, Cary, NC) with 488 nm excitation and 525 nm emission. Six independent measurements were taken for each PI concentration ($n = 6$). To measure chondrocyte-generated ROS by the photoinitiation process, single donor chondrocytes (non-plated or plated) in suspension culture were incubated with 20 μM carboxy-H_2DFFDA for 20 min to allow for its transport into cells and subsequent cleavage of the diacetate followed by several rinses in PBS via centrifugation. Cells (4×10^6 cells/mL) were resuspended in chondrocyte medium with 0, 1, 2, 4.5 or 11 mM photoinitiator and transferred to 96 well plates (80 μl/well). Wells were either left untreated or exposed to 365 nm light and fluorescence immediately assayed on the plate reader as described above. Three biological replicates were performed with two technical replicates per experiment ($n = 3$). In a separate experiment, intracellular generation of photoinitiator radicals and/or ROS resulting from photoinitiator molecules being taken up by cells followed by ultraviolet (UV) exposure was investigated in non-plated cells (termed photodynamic effect). Pooled donor chondrocytes were incubated with 0–11 mM photoinitiator for 20 min to allow cells to take up the photoinitiator molecules. Cells were rinsed in PBS by centrifugation, treated with carboxy-H_2DFFDA and UV light, and assessed by fluorescence as previously described. Two biological replicates were performed with two technical replicates per experiment ($n = 2$). In 3D culture, single donor chondrocytes (non-plated or plated) were incubated with carboxy-H_2DFFDA for 20 min, rinsed in PBS by centrifugation, and encapsulated in PEG hydrogels as described above directly in 96 well plates. Immediately post-encapsulation, fluorescence was assessed on the plate reader or imaged on a confocal laser scanning microscope (CLSM). Three biological replicates were performed with three technical replicates per experiment ($n = 3$).

Quantification of malondialdehyde (MDA)

MDA is a stable byproduct of the reaction between high-energy radicals and lipids in the cell membrane. Single donor chondrocytes from plated and non-plated conditions were encapsulated in PEG hydrogels as described above and cultured up to 3 days. At prescribed times, gels were snap frozen and homogenized in lysis buffer (20 mM Tris, 2 mM EDTA, 150 mM NaCl and 0.5% Triton X-100 in deionized water, Sigma–Aldrich). MDA content was determined using a thiobarbituric reactive species assay kit (Cayman Chemical, Ann Arbor, MI) and FLUOstar Optima plate reader per manufacturer. Three biological replicates were performed with three technical replicates per experiment ($n = 3$).

Gene expression

Single donor chondrocytes from plated and non-plated conditions were encapsulated in PEG hydrogels as described above and cultured for 2 weeks. At prescribed times, gels were snap frozen and homogenized in TRK lysis buffer (Omega Bio-Tek, Norcross, GA) with 2% β-mercaptoethanol (Sigma–Aldrich). Samples were eluted through a homogenizer column (Omega Bio-Tek) at 13,000 rpm for 5 min to separate PEG from lysate. RNA was extracted by centrifugation using micro-elute RNA binding columns (Omega Bio-Tek) per manufacturer. RNA was converted to cDNA using high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA). All primers (Table I) were designed to span an entire intron (Integrated DNA Technologies, San Diego, CA). Quantitative polymerase chain reaction (qPCR) was performed (7500 Fast real-time PCR

Table I
Primer sequences designed for use in real-time PCR gene expression analysis

Gene of interest	Forward primer	Reverse primer
Aggrecan	5′-GCGGGTGCGGGTCAA-3′	5′-ATCACCTCGCAGCGTAGAATC-3′
Collagen II	5′-AGTCCCTCAACAACC AGATCG-3′	5′-CGATCCAGTAGTCTCCGCTCTT-3′
Collagen VI	5′-GGCCGGACTCCACTGAGA-3′	5′-TCTCCATAGGCTTCATG TTTCATG-3′
MMP-13	5′-TCCTGGCTGGCTTCCTCTT-3′	5′-GTAGCTCTCTGCAAACTG GAAGTCT-3′

system, Applied Biosystems). Normalized gene expression is defined as[26]

$$\text{Normalized expression} = \frac{(E_T)^{\Delta C_{TT}(\text{calibrator}-\text{test})}}{(E_R)^{\Delta C_{TR}(\text{calibrator}-\text{test})}}$$

where E_T and E_R are the efficiencies of the target and housekeeping genes, respectively, C_{TT} and C_{TR} are the difference in C_T values between calibrator and test sample for the target and housekeeping genes, respectively. The ribosomal protein L30 was used as the stable housekeeping gene. Calibrator was day 0. Three biological replicates were performed with two technical replicates per experiment ($n = 3$).

Proteoglycan synthesis

Proteoglycan synthesis was assessed in PEG hydrogels encapsulated with pooled donor chondrocytes from non-plated and plated conditions and cultured in chondrocyte medium supplemented with 10 μCi/mL $^{35}SO_4$ (Perkin Elmer, Shelton, CT) for 24 h

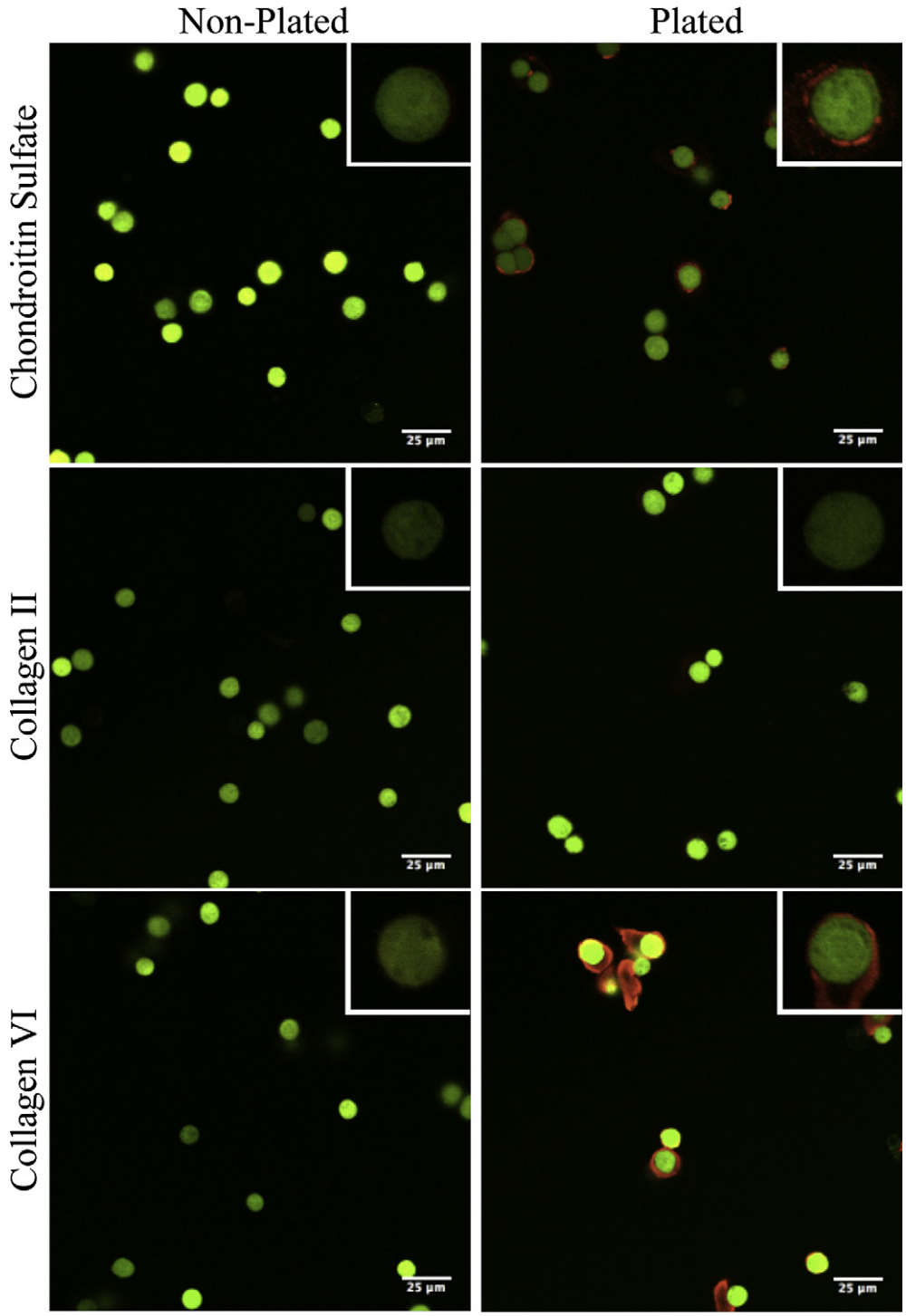

Fig. 2. The presence of PCM in live chondrocytes from non-plated and plated conditions encapsulated in PEG hydrogels. The PCM molecules examined were chondroitin sulfate, collagen II and collagen VI (red) by IC, while the cytosol of the live cells was counterstained with calcein AM (green) ($n = 1$ biological replicate).

prior to analysis. At prescribed times, medium was removed for analysis and hydrogels were homogenized and enzymatically digested (125 μg/mL Papain (Worthington), 10 mM L-cysteine, 100 mM phosphate and 10 mM EDTA (Sigma–Aldrich) in DI water (pH 6.3)) overnight at 60°C. Newly synthesized proteoglycans were assayed by Alcian blue precipitation.[27] Briefly, Alcian blue (0.2% w/v) was added to media and construct digest samples, which binds to and precipitates proteoglycans. The precipitate was captured by

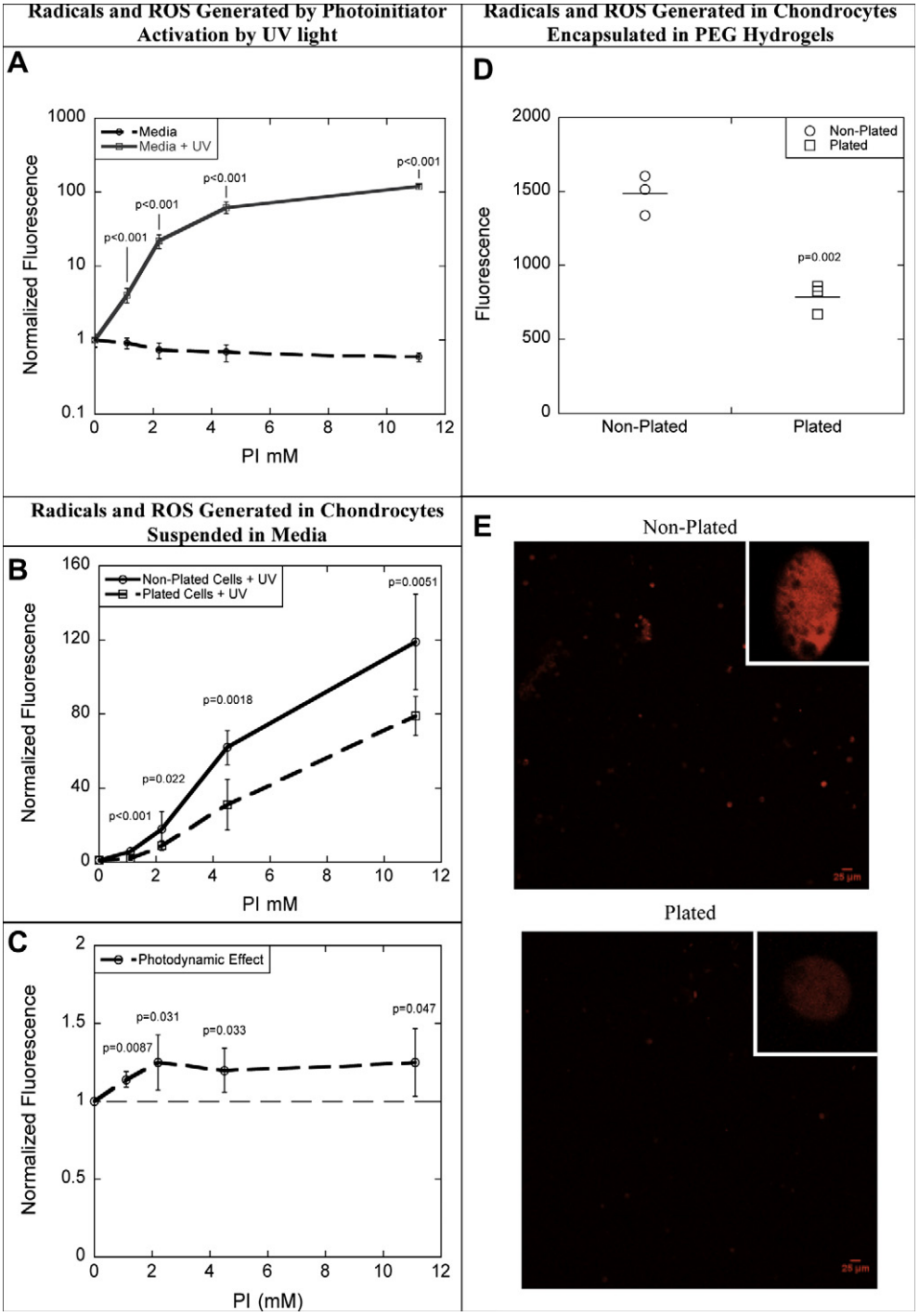

Fig. 3. ROS generation as measured by fluorescence using carboxy-H_2DFFDA. (A) ROS generated by photoinitiator molecules in media with and without UV exposure, where fluorescence was normalized to samples with no photoinitiator ($n = 6$). (B) ROS generated intracellularly in chondrocytes from non-plated and plated conditions, which were suspended in media and exposed to UV light. Fluorescence was normalized to samples with no photoinitiator but exposed to UV ($n = 3$ biological replicates). (C) ROS generated in chondrocytes from non-plated condition resulting from cells taking up photoinitiator molecules and subsequent exposure to UV light (photodynamic effect). Fluorescence was normalized to samples with no photoinitiator. The dashed line represents samples with no PI but with UV exposure ($n = 2$ biological replicates). (D) ROS generation intracellularly in chondrocytes immediately after photoencapsulation in PEG hydrogels. (E) Confocal microscopy images of intracellular ROS generation (red) immediately after photoencapsulation of chondrocytes in PEG hydrogels ($n = 3$ biological replicates). In A–C, data are presented as the mean and error bars represent the 95% confidence interval of the mean. *P* values represent significant differences between experimental conditions for a given PI concentration. In D, data points from non-plated and plated conditions are presented. *P* values represent significant differences from the non-plated condition.

filtration (Multiscreen HTS Filter Plate, Fisher Scientific, Pittsburgh, PA), and released by incubation with 1.34 g/mL guanidine-HCl (Sigma) in deionized water/isopropanol solution (2:1 v/v) (Sigma). Total proteoglycans were quantified in cpm on a Beckman LSC 6500 (MDA 80.3 pCi) and normalized to gel wet weight. Two biological replicates were performed with three technical replicates per experiment ($n = 2$).

Statistical analysis

Data are reported as the mean of the biological replicates or as individual data points in a dot plot. Error bars represent 95% confidence intervals of the mean. One-way analysis of variance was used with Tukey's post-hoc analysis and $P < 0.05$ considered significant. All data followed a Gaussian distribution and exhibited homogeneous variance. The analysis unit for each experiment was cells in solution or encapsulated, either from one animal or from several animals pooled together, which were subjected to a treatment (e.g., photoinitiator, UV exposure) applied at random.

Results

Freshly isolated chondrocytes were largely devoid of a PCM evidenced by a lack of staining for chondroitin sulfate and collagens II and VI, as shown in Fig. 2. A 24-h plating period, however, was sufficient for chondrocytes to re-form some of their own PCM comprised of chondroitin sulfate and collagen VI, although collagen II was not yet present (Fig. 2).

When photoinitiator molecules dissolved in chondrocyte medium in the absence of cells were exposed to a single dose of UV light in the presence of oxygen and hydrolyzed carboxy-H_2DFFDA, fluorescence increased significantly with increasing photoinitiator concentration [Fig. 3(A)]. Fluorescence was significantly higher for each corresponding photoinitiator concentration without UV exposure. For example, fluorescence was 268-fold higher ($P < 0.001$) for the 11 mM photoinitiator sample with UV exposure than without UV exposure.

When cells were present in solution, photoinitiator radicals led to elevated intracellular ROS, which was a function of photoinitiator concentration and dependent on cell plating condition [Fig. 3(B)]. Non-plated conditions had 1.5–3-fold higher ($P < 0.001$) intracellular ROS levels for photoinitiator concentrations of 1.1 mM and greater, respectively over plated conditions. For non-plated cells, transport of photoinitiator molecules into cells and subsequent intracellular ROS generation by UV light (photodynamic effect) was assessed [Fig. 3(C)]. The photodynamic effect resulted in significantly higher intracellular ROS when compared to untreated cells exposed to UV light for all photoinitiator concentrations. Specifically, fluorescence increased by 1.25-fold at 2 mM photoinitiator and remained constant with higher photoinitiator concentrations.

When cells were photoencapsulated in PEG hydrogels with 2 mM photoinitiator, non-plated conditions showed 1.89-fold higher ($P = 0.002$) levels of intracellular ROS when compared to plated conditions [Fig. 3(D)]. Qualitatively, confocal microscopy images also showed higher levels of intracellular ROS immediately post-encapsulation in non-plated over plated conditions [Fig. 3(E)].

Damage to the cell membrane as a result of photoencapsulation was assessed by MDA content (Fig. 4). Immediately post-encapsulation, non-plated conditions in PEG had 1.45-fold higher ($P = 0.016$) MDA content when compared to plated conditions in PEG and 1.69-fold higher in non-plated conditions in agarose ($P = 0.0041$), both of which had similar MDA levels. By day 3 MDA levels were similar among conditions in PEG.

Relative expression of anabolic and catabolic genes in PEG hydrogels encapsulated with chondrocytes from non-plated or plated conditions is shown in Fig. 5. All anabolic genes examined (aggrecan, collagen II and collagen VI) decreased ($P < 0.001$) during the first week of culture for both non-plated and plated conditions. During week two, all anabolic genes increased over time. Plated condition, however, led to generally higher gene expression levels by 0.83–920-fold for all anabolic genes when compared to non-plated conditions over the 2 weeks. Relative expression for the catabolic gene MMP-13 did not significantly change over the 2-week period for the plated condition, but was 54.7% lower ($P < 0.001$) on average for the non-plated condition compared to the plated condition.

Proteoglycan synthesis was measured by $^{35}SO_4$ incorporation into newly synthesized GAGs (Fig. 6). Proteoglycan synthesis was 25-fold higher ($P < 0.001$) for the plated condition 1-day post-encapsulation over the non-plated condition. By days 3 and 5, synthesis rates were comparable.

Discussion

This study confirms that chondrocytes incur oxidative stress during photoencapsulation, evidenced by increased intracellular ROS and lipid peroxidation. The presence of PCM partly mitigates polymerization-induced oxidative stress leading to higher anabolic gene expression and improved tissue synthesis in bovine chondrocytes when photoencapsulated in PEG hydrogels. Our findings suggest that PCM serves as a natural defense mechanism against radicals and polymerization-induced ROS, thus protecting chondrocytes during photoencapsulation.

During photoencapsulation, chondrocytes are exposed to radicals generated by the photopolymerization reaction and by extracellular ROS generated from photoinitiator radicals reacting with oxygen. Both radicals and ROS have damaging effects on chondrocytes[15,28–30] and our findings provide evidence of increased intracellular ROS in chondrocytes when exposed to photoinitiator molecules and light, i.e., the photoinitiation step of photopolymerization. We postulated that intracellular ROS generation may result from several possible modes of action: (1) photoinitiator molecules are taken up by cells and upon activation by

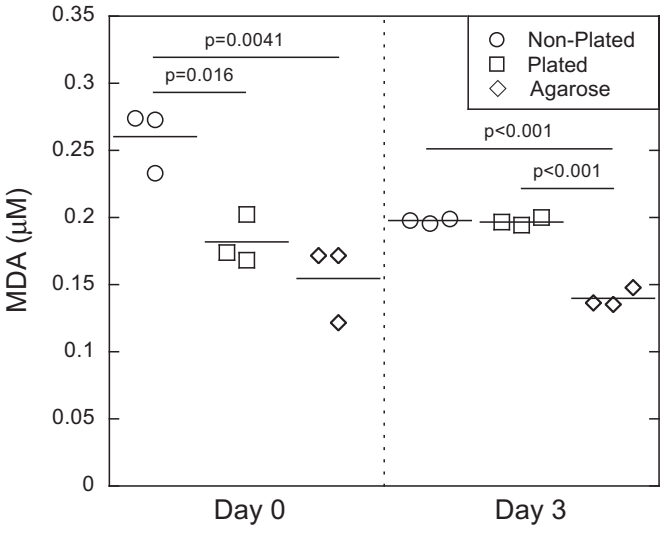

Fig. 4. Detection of lipid peroxidation by MDA content in chondrocytes from non-plated (without PCM) and plated (with PCM) conditions after photoencapsulation in PEG hydrogels and in non-plated chondrocytes (without PCM) encapsulated in agarose hydrogels. Data points for each condition (non-plated, plated, and agarose) and time point are presented from three independent experiments ($n = 3$ biological replicates). *P* values represent significant differences between conditions for a given time point.

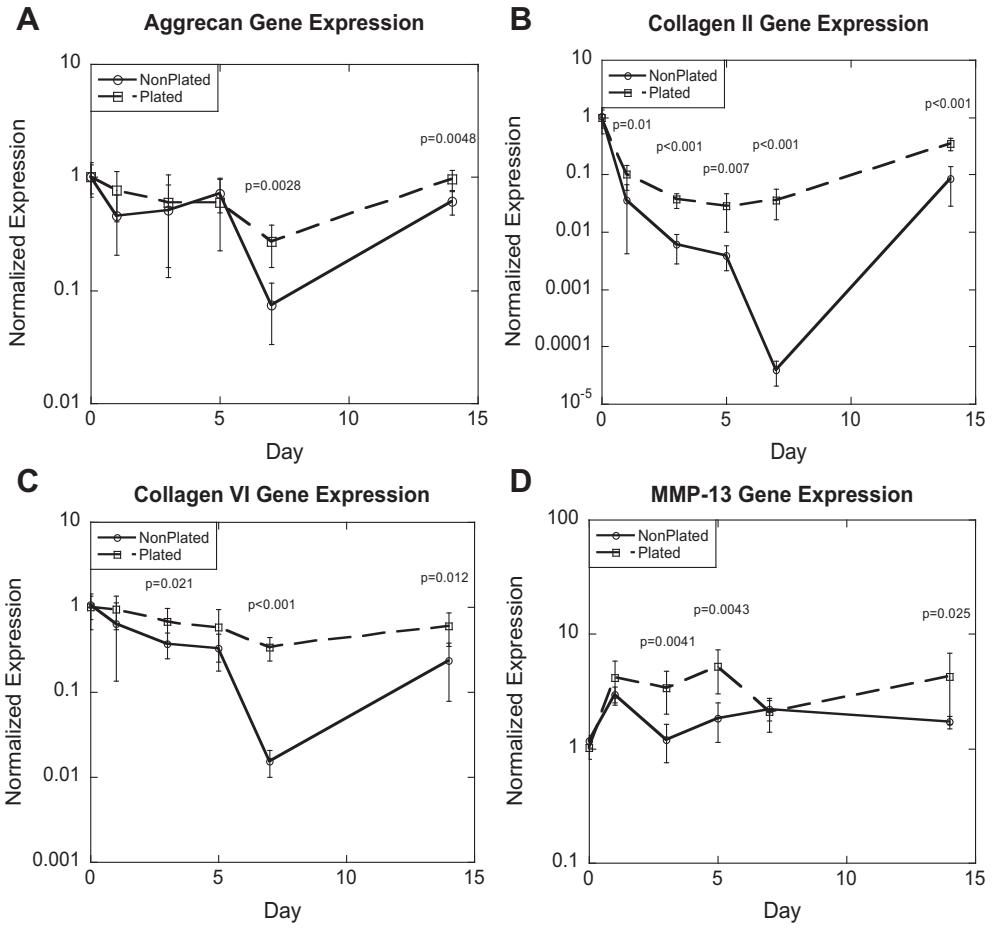

Fig. 5. Normalized gene expression for (A) aggrecan, (B) collagen type II, (C) collagen type VI, and (D) MMP-13 in chondrocytes from non-plated (without PCM) and plated (with PCM) conditions when photoencapsulated and cultured in PEG gels. Normalized expression represents relative expression for each gene normalized to the average expression at day 0 for the respective condition. Data are presented as the mean and error bars represent the 95% confidence interval of the mean from three independent experiments ($n = 3$ biological replicates). *P* values represent significant differences between experimental conditions for a given time point.

UV light react with intracellular oxygen produce intracellular ROS (photodynamic effect), (2) extracellular ROS generated during photoinitiation diffuses into cells and is detected as intracellular ROS, and (3) ROS produced by the polymerization and/or radicals formed during polymerization react with cells, e.g., the cell membrane, leading to downstream events that generate intracellular ROS.

To address the first mode, our findings investigating the photodynamic effect indicated that only a small fraction of photoinitiator molecules is taken up by chondrocytes causing intracellular ROS generation upon UV exposure, accounting for ~1–9% (depending on photoinitiator concentration) of the total intracellular ROS. As carboxy-H_2DFFDA may be oxidized by both photoinitiator radicals and ROS, we cannot distinguish between photoinitiator radicals and ROS generated within the cell. Nonetheless, these findings suggest that the photodynamic effect is minimal.

For the second mode to occur, stability of radicals and ROS must be considered. Radicals have limited distances over which they can diffuse due to their short half-life, while more stable non-radical based ROS can diffuse over considerable distances and readily enter cells. This observation suggests that only a small fraction of ROS or photoinitiator radicals are likely to diffuse into cells, but that any stable ROS, e.g., hydrogen peroxide, which may form through subsequent reactions, could enter cells. Differences can be inferred between levels of radicals and ROS generated by the photoinitiation reaction extracellularly and levels of intracellular ROS. With the volume fraction of cells being ~1%, similar levels of fluorescence detected under both scenarios indicate that intracellular ROS concentrations are likely substantially higher than radical/ROS concentration outside the cell. This observation points toward the last proposed mode of action, suggesting radicals and/or ROS generated by photoinitiation damage cells initiating downstream events that trigger intracellular ROS generation.

Indeed, photopolymerization-induced lipid peroxidation was confirmed by a greater presence of MDA[31,32] in cells that were photoencapsulated. Others have shown that lipid peroxidation leads to intracellular ROS generation in other cell types.[33,34] Lipid peroxidation requires highly reactive species and only certain radicals/ROS are capable of reacting with membrane lipids.[35] Possible candidates formed during photopolymerization include photoinitiator radicals, macroradicals formed during propagation, and highly ROS, such as peroxy radicals, which are formed as a result of the photopolymerization reaction occurring in the presence of oxygen. Photopolymerization-induced lipid peroxidation was not sustained and by day 3 MDA levels were similar to agarose, suggesting a return to basal levels of lipid peroxidation, which has been observed in 2D chondrocyte cultures.[36] Taken together, our findings point toward the hypotheses that intracellular ROS generated in chondrocytes during photoencapsulation results from direct damage to the cell, triggering intracellular ROS generation, and to a lesser extent by extracellular photoinitiator radicals and ROS diffusing into chondrocytes.

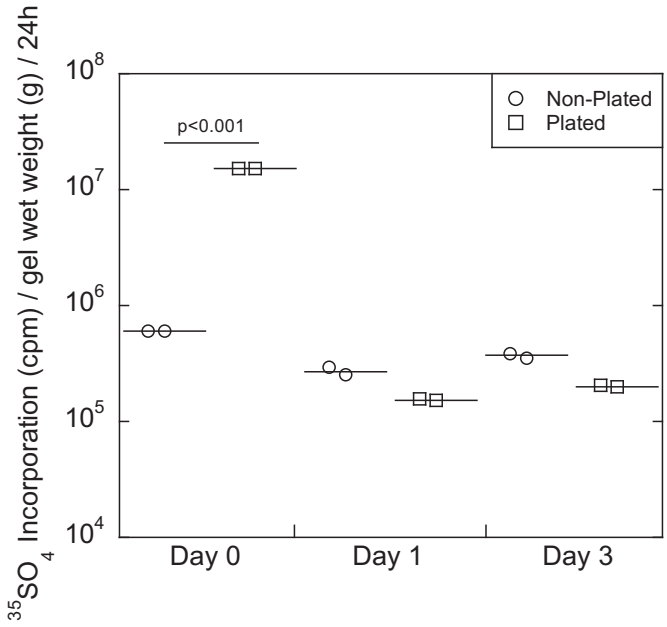

Fig. 6. Proteoglycan synthesis measured by $^{35}SO_4$ incorporation over a 24 h period in chondrocytes from non-plated (without PCM) and plated (with PCM) conditions when encapsulated and cultured in PEG gels as measured in counts per minute (cpm) and normalized to the wet weight of the construct in grams. Data points for each condition (non-plated and plated) and time point from two independent experiments are presented ($n = 2$ biological replicates). *P* values represent significant differences from the non-plated condition for a given time point.

When chondrocytes formed some of their own PCM prior to photoencapsulation, intracellular ROS generation was significantly reduced by ~40% and the level of lipid peroxidation induced by photoencapsulation was partially abrogated. This observation suggests that radicals and/or extracellular ROS generated during photoencapsulation may diffuse into cells and that the presence of the PCM can reduce, but not abrogate this mode of action. It is well known that proteins and other matrix molecules can react with and terminate radicals.[10,37] Nascent PCM was comprised of chondroitin sulfate, one of the main building blocks of aggrecan, and collagen VI, the main collagen in the chondrocyte PCM.[38] Of the molecules found in cartilage PCM, hyaluronan a building block of aggrecan is most well known for its antioxidant properties, acting as radical scavengers.[39] While hyaluronan was not explicitly detected in the nascent PCM, the presence of chondroitin sulfate suggests that aggrecan and hyaluronan are present.[40] This property, along with the ability of the PCM to act as a physical barrier between the extracellular environment and the cell membrane, appears to have reduced radical damage to membrane lipids during photoencapsulation and reduced intracellular ROS.

While photoinitiation alone leads to oxidative stress, it is important to recognize that during photoencapsulation, the concentration of radicals rises exponentially. This effect, referred to as auto-acceleration, occurs because mobility of large macroradicals becomes increasingly more limited, while macromers can readily diffuse and continue propagation.[1,11] For photoinitiator concentrations typically employed for cell encapsulation (i.e., 2 mM), our data show that intracellular ROS generated during photoencapsulation is significantly higher than for cells simply exposed to photoinitiator radicals [i.e., comparing Fig. 3(B) at 2 mM with 3E]. This finding suggests that in addition to photoinitiator radicals, macroradicals generated during polymerization also have damaging effects on cells leading to oxidative stress.

Oxidative stress in chondrocytes is known to have downstream effects, negatively impacting their metabolism and inducing tissue destruction.[16,36,41] In this study, photoencapsulation of chondrocytes lacking a PCM exhibited reduced anabolic gene expression and reduced matrix synthesis when compared to chondrocytes with a PCM. This finding suggests that oxidative stress incurred by chondrocytes during photoencapsulation may have longer-term negative effects on cell metabolism, which is supported by other studies. For example, induction of ROS in cartilage explants led to decreased collagen II and aggrecan gene expression and collagen II production.[42] *Ex vivo*, hydrogen peroxide treatment of cartilage explants induced lipid peroxidation and enhanced proteolytic activity.[33] ROS is closely linked to osteoarthritis and is thought to upregulate catabolic enzymes and induce tissue degradation in osteoarthritic cartilage.[37] In this study, catabolic activity, specifically gene expression of MMP-13, which is prevalent in osteoarthritic cartilage,[34] decreased over time in chondrocytes lacking PCM during photoencapsulation. While oxidative stress has been shown to upregulate MMP-13 activity in chondrocytes,[16,43] exposure to radicals may be too short (i.e., on the order of minutes) to induce a catabolic response longer-term. In contrast osteoarthritic chondrocytes are exposed to ROS for prolonged periods.[37] It is possible that radicals and ROS may affect post-translational MMP-13 activity, which was not evaluated in this study. Interestingly, MMP-13 gene expression in chondrocytes encapsulated with a PCM was greater than without a PCM. In non-degrading PEG hydrogels the relatively tightly cross-linked network limits matrix elaboration to the pericellular space.[43] With increased matrix deposition in the plated condition, there may be a greater need for matrix turnover[44] where MMP-13 may be involved.[45] Future studies are needed to confirm these hypotheses. Nonetheless, our findings confirm the presence of PCM prior to photoencapsulation improves chondrocyte anabolism longer-term.

In conclusion, the photoencapsulation process is known to lead to the generation of photoinitiator radicals and macroradicals on propagating chains and to extracellular ROS when oxygen is present, which induce oxidative stress in chondrocytes. This oxidative stress appears to have longer-term negative effects on chondrocyte anabolism. The presence of a PCM, however, reduces the level of oxidative stress and improves chondrocytes anabolic activity. Our findings suggest that minimizing oxidative stress, such as through the presence of PCM, may have long-term beneficial effects on tissue elaboration when employing photopolymerizations to encapsulate chondrocytes for cartilage tissue engineering applications.

Author contributions

NF: Design of the study, acquisition and interpretation of data, drafting, revising and final approval of the article.

CB: Design of the study, acquisition and interpretation of data, drafting and final approval of the article.

SJB: Design of the study, interpretation of data, drafting, revising and final approval of the article.

Conflict of interest

The authors report no conflicts of interest.

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